

Aus dem
CharitéCentrum für Tumormedizin
Med. Klinik mit Schwerpunkt Hämatologie, Onkologie und Tumorimmunologie
Direktor: Prof. Dr. Lars Bullinger

Habilitationsschrift

A single cell approach to tumor-infiltrating lymphocytes in solid and hematopoietic malignancies

zur Erlangung der Lehrbefähigung
für das Fach Innere Medizin

vorgelegt dem Fakultätsrat der Medizinischen Fakultät
Charité-Universitätsmedizin Berlin

von

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Eingereicht:	April 2019
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1. Abbreviations (Abkürzungen)

activation-induced cytidine deaminase (AID)	monoclonal gammopathy of undetermined significance (MGUS)
B-cell activating factor belonging to the tumor necrosis family (BAFF)	naturally occurring regulatory T cell (nTreg) nuclear factor of activated T cells (NFAT)
chimeric antigen receptor (CAR)	pathogen-associated molecular pattern (PAMP)
committed lymphoid progenitors (CLP)	peripheral blood mononuclear cells (PBMCs)
complementarity determining region (CDR)	Phorbol Myristate Acetate (PMA)
Cytometry by Time-of-Flight (CyTOF)	plasmacytoid dendritic cells (pDCs)
cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4)	programmed cell death protein 1 (PD-1)
diversity region (D)	recombination activating gene (RAG)
double negative (DN)	regulatory T cell (Treg)
epidermal growth factor receptor (EGFR)	RNA sequencing (RNAseq)
forkhead box P3 (FOXP3)	Src homology region 2-containing protein tyrosine phosphatase 2 (SHP2)
graft versus host disease (GvHD)	T cell immunoglobulin domain and mucin domain-containing protein 3 (TIM-3)
human epidermal growth factor receptor 2 (HER2)	T cell receptor (TCR)
human leukocyte antigen (HLA)	T helper 1 (Th1)
induced regulatory T cell (iTreg)	toll-like receptor (TLR)
interferon (IFN)	transforming growth factor (TGF)
interleukin (IL)	Treg-specific demethylated region (TSDR)
joining region (J)	tumor necrosis factor (TNF)
killer cell lectin-like receptor subfamily G member 1 (KLRG1)	tumor necrosis factor receptor associated factor 1 (TRAF1)
lymphocyte activation gene 3 (LAG-3)	tumor node metastasis (TNM)
major histocompatibility complex (MHC)	tumor-infiltrating T cell (TIL)
	Union for International Cancer Control (UICC)
	variable region (V)

2. Introduction (Einleitung)

This work is focused on human immunology in solid and hematopoietic malignancies. In the beginning of the 21st century, tumor immunology was a relatively small field often confronted with the dogmatic opinion that the immune system was made for protection from exogenous antigens and therefore would not be capable of controlling cancer. However, the development of monoclonal antibodies targeting cancer-associated antigens, immune checkpoint blockade, and clinical application of genetically engineered immune cell products have improved clinical outcomes of patients, won Nobel prizes, and attracted profound medical, scientific, and economic interest to the now rapidly expanding field of tumor immunology. Despite enormous success, cancer immunotherapy has only been effective in subsets of patients and the underlying mechanisms are incompletely understood.

This work presents the development of novel methodologies in human T and B cell immunology leading to the identification of mechanisms of tolerance induction and tumor-directed immune responses in rectal cancer and multiple myeloma as disease models for solid and hematopoietic malignancies.

Tumor immune infiltrates have critical impact on prognosis and survival of rectal cancer patients whereas in multiple myeloma, the immune system, especially the B lineage, gives rise to the malignant cells. Understanding the roles of immune cells in the context of cancer requires knowledge of basic principles of T and B cell development, differentiation, and induction of immunity and tolerance. Tolerogenic cells, such as regulatory T cells, in the tumor microenvironment are generally perceived as predictors of negative outcome possibly interfering with tumor-directed immune responses. Therefore, it is critical to understand mechanisms that lead to the induction of inflammation and immunity as much as tolerance to

identify characteristics of immune cells that can potentially be re-programmed and support processes leading to cancer control or eradication.

Human tumor immunology can only be reliably studied in primary human specimens. To account for substantial heterogeneity inherently connected with human subjects, high-dimensional technologies and analysis strategies at the single cell level are required. The introduction will give an overview of T and B cell maturation and differentiation, which is critical to understand the developed methodologies and conclusions drawn from our data. The following selected publications (chapter 3) will introduce methodologies for i) the isolation of intact nucleic acids after intranuclear staining [1], and ii) definition of specificity, phenotype, and function of lymphocytes at the single cell level by fluorescence activated single cell index sorting and deep sequencing [2-4]. The technological spectrum is completed with mass cytometry (or Cytometry by Time-of-Flight, CyTOF), which allows the definition of the expression of more than 30 markers on single cells in a high-throughput fashion. These technologies build the foundation for in-depth interrogation of the tumor microenvironment and interrelatedness of single lymphocytes in rectal cancer and multiple myeloma in the second part of this work [4-6].

2.1 T cell maturation

Mature T cells are characterized by the expression of $\alpha\beta$ or $\gamma\delta$ T cell receptors (TCR) in combination with either CD4 or CD8 co-receptors.

T cells arise from committed lymphoid progenitors (CLP) in the bone marrow and migrate to the thymus for major parts of their maturation. CLP enter the thymus and, after losing potential to differentiate into B or NK cells, define the pool of double negative (DN, $CD4^-CD8^-$) committed T cell precursors [7-9]. DN T cells pass through four different stages of development (DN1-4) and can still differentiate into either $\alpha\beta$ or $\gamma\delta$ T cells [10]. $\alpha\beta$ T cells express TCR consisting of α and β chains that determine specificity and recognize antigen in

complex with major histocompatibility complex (MHC, or human leukocyte antigen, HLA) class I or II. TCRs of $\gamma\delta$ T cells are heterodimers of γ and δ chains and can recognize antigen independent from MHC. This work is focused on $\alpha\beta$ T cells, in the following called T cells unless otherwise stated.

The TCR is one of the most diverse structures in the human body, unique for each T cell clone, and determines specificity. Diversity is encoded in the TCR $\alpha\beta$ genes and created by a specialized DNA editing process, V(D)J recombination (see chapters 2.2 and 2.8), which leads to TCR sequences unique for each individual T cell clone. In DN3 and DN4 stages, recombination activating gene (RAG)1 and 2 are required for rearrangement of first the TCR β and subsequently the TCR α locus (see chapter 2.8) [11, 12]. Intermittent expression of a pre-TCR [13] prevents successful rearrangement of the second TCR β allele (allelic exclusion) [14-16]. Once fully rearranged TCR $\alpha\beta$ /CD3 complexes have been formed, T cells start to express co-receptors, such as CD8 and CD4 (usually CD8 followed by CD4) resulting in a population of double positive CD4⁺CD8⁺ immature T cells that can take up to 90 % of the thymic lymphoid compartment in young individuals [10]. The mechanisms determining whether T cells develop into CD4⁺CD8⁻ T helper or CD8⁺CD4⁻ cytotoxic T cells have been under debate [17-22]. The CD4/CD8 decision is critical as a particular TCR can only recognize its antigen in complex with the compatible MHC and co-receptors. While recognition of antigen in complex with MHC class I requires CD8 expression, CD4⁺ T cells can only recognize antigen in complex with MHC class II. This MHC-tropism does not only reduce the number of potential partners for interaction through the TCR but also restricts the type of target antigens. A T cell's CD4/CD8 choice is not entirely stochastic but assumed to be influenced by strength and duration of signaling through the TCR in the thymus, cytokine composition in the microenvironment, and expression of nuclear factors [23].

2.2 Creation of the T cell receptor

The immune system is constantly challenged by an almost infinite variety of pathogens asking for equally flexible mechanisms to generate a pool of highly diverse T cell specificities. Specificity is encoded in the T cell receptor α and β gene sequences [24-27]. The TCR recognizes antigen in form of short peptides bound to MHC class I or class II [18] and contains three complementarity determining regions (CDR). The third hypervariable region (CDR3) forms the TCR antigen binding site. To ensure availability of T cells with almost any specificity, diversity in the CDR3 of TCR α and TCR β genes is introduced by irreversible DNA recombination of variable (V), diversity (D), and joining (J) regions.

The process of V(D)J recombination was first discovered in B cells in the 1970-ies [28-32] before similar sets of genes and mechanisms of recombination were identified to account for variability of T cell receptor genes [33]. The elements of the TCR genes (V, D, J segments) include 50 V and 70 J segments for the α chain located on chromosome 14 and 57 V, 2 D, and 13 J segments for the β chain on chromosome 7. While recombination of the TCR β locus involves V, D, and J segments, the TCR α locus only comprises of V and J segments [34]. Each V, D, and J segment is flanked by DNA-encoded recombination signals recognized by RAG-1 and 2 – the key enzymes in VDJ recombination for both the TCR and B cell receptor genes [35]. At the TCR β and the immunoglobulin heavy chain locus, D to J recombination precedes V to DJ recombination. Since each cell contains two alleles for the TCR α and β loci, reliable mechanisms are required to prevent VDJ recombination of the second allele once a productive TCR α or β rearrangement has been formed to ensure each T cell expresses exactly one TCR (allelic exclusion). Regarding the TCR β gene, D and J segments are usually rearranged on both alleles, however, productive VDJ recombination only occurs on one allele. The fully rearranged TCR β forms the pre-TCR in complex with CD3 and a germline encoded pre-TCR α [36]. It is generally accepted that signaling through the pre-TCR leads to downregulation of

RAG-1/2 gene expression, transition to the double-positive stage of T cell development, and inhibition of the second V to DJ rearrangement enabling allelic exclusion [37].

Combinatorial joining of V, (D), and J segments results in theoretically approx. 1000 possible variants for TCR α and more than 2500 for TCR β [38]. Besides the theoretically possible combinations, V, (D), and J segments are enzymatically modified during rearrangement (N region variability) [38, 39] leading to an almost unlimited diversity of the TCR repertoire and providing unique molecular identifiers for each individual T cell clone – the TCR $\alpha\beta$ CDR3 sequences.

2.3 Positive and negative T cell selection

The almost infinite variety of possible TCRs resulting from V(D)J recombination requires mechanisms that warrant elimination or silencing of T cells with relevant self-reactivity to prevent autoimmunity. These mechanisms ensure that i) T cells can bind antigen in complex with autologous MHC class I or II, ii) self-antigens are sufficiently presented during thymic selection not only by MHC class I but also class II, and iii) potentially autoreactive T cells are being eliminated or sustainably programmed towards tolerance.

Major parts of these critical processes take place in the thymus and include “positive selection”, “negative selection”, and “death by neglect”. Positive selection refers to stimulation of immature double-positive thymocytes through intermediate-affinity TCR-peptide-MHC interactions resulting in proliferation and differentiation into mature single-positive T cells. Negative selection, also known as clonal deletion, is the process of elimination of thymocytes expressing TCRs with high affinity for self-antigens. Thymic deletion of high-affinity self-reactive T cells is considered critical in the maintenance of tolerance and, due to thymic localization, called “central tolerance”. Death by neglect refers to induction of apoptosis within thymocytes that fail to engage in positively selecting interactions due to multiple reasons, e.g. inability to bind to MHC.

Positive and negative selection of T cells and their functional programming result from complex interactions of T cells with antigen-presenting cortical thymic epithelial cells, medullary thymic epithelial cells, dendritic cells, and B cells, among others [40, 41]. The majority of immature T cells does not undergo successful positive selection; negative selection and death by neglect account for the loss of approx. 90 % of T cell precursors.

Thymic T cell selection processes appear to follow spatially restricted patterns. While cortical thymic epithelial cells are mostly involved in positive selection, presentation of endogenous antigens by medullary thymic epithelial cells is critical for negative selection of CD8⁺ T cells and induction of CD4⁺ T cell tolerance [42, 43]. The presentation of endogenous antigens to CD4⁺ T cells is especially challenging as CD4⁺ T cells recognize antigen bound to MHC class II, however, MHC class II usually presents exogenous, e.g. phagocytosis-derived antigens. Although autophagy is assumed to have a major role in MHC class II presentation of autoantigens, especially membrane proteins seem to access MHC class II independently of autophagy [44, 45]. Most antigen presentation in the thymus takes place on cortical or medullary epithelial cells. Dendritic cells – the major professional antigen-presenting cells in the periphery – account for approx. 0.3 % of thymic cells and are predominantly located in the medullary part of the thymus. Plasmacytoid dendritic cells (pDCs) account for 1/3 of thymic DCs and, while having a minor role for antigen presentation in the periphery, have been shown to efficiently present antigen to thymocytes and promote differentiation into regulatory T (Treg) cells [46, 47].

Interestingly, activation of toll-like receptors (TLRs) prevents all subsets of DCs from migrating into the thymus. TLRs are involved in sensing and subsequently responding to pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), double-stranded DNA (dsDNA), and others. This mechanism is supposed to prevent the induction of central tolerance to pathogens under inflammatory conditions [48, 49].

B cells account for approx. the same proportion of thymic cells as DCs (0.3 %). Although they have been shown to effectively induce negative selection of CD4⁺ T cells [50], they are not assumed to negatively select CD8⁺ thymocytes [51]. Besides their ability to present self and foreign antigens, the presence of B cells in the thymus may be critical for maintaining tolerance towards variable regions of the B cell receptor genes, a unique class of self-antigens [52].

Between positive and negative selection remains a stochastic overlap resulting in the survival of T cells with relatively high affinity self-reactive TCRs, which, at least in parts, differentiate into natural regulatory T cells. However, central tolerance cannot completely eradicate self-reactive T cell clones suggesting mechanisms other than positive and negative selection to maintain tolerance in the periphery.

2.4 Determination of T cell function

While T cell antigen specificity is defined by the TCR, T cell function is determined by immune phenotype, cytokine, and transcription factor profiles. A variety of surface markers have been identified for the definition of T cell subsets with more or less well defined functional properties. It is generally accepted that by determining the expression of CD4, CD8, CCR7, and CD45RA, T cells can be categorized into naïve, central memory, effector, and effector memory T cells [53]. Naïve T cells are assumed to have not yet encountered target antigen in the periphery, whereas memory T cells represent the pool of T cells that remain after contraction from antigen-driven expansion ready to proliferate again upon antigen re-exposure [54, 55].

Initially, T cell function and differentiation had been defined by surface molecule expression and cytokine production leading to the categorization of T cells into different lineages. For example, T helper 1 (Th1) cells were defined by the production of interferon (IFN)- γ , interleukin (IL)-2, and tumor necrosis factor (TNF)- β whereas IL-4 and IL-13 represent key cytokines of Th2 differentiation [56, 57]. However, cytokine profiles of individual populations

were almost never homogenous. With the availability of high-dimensional gene expression technologies, it has been well established that especially CD4⁺ Th1, Th2, Th17, and Treg (among others) express and can additionally be defined by lineage-defining transcription factors [58-61].

Initially, it was assumed that a T cell, once committed to a certain lineage (Th1, Th2, ...), was functionally defined and maintained its differentiation state [62]. The concept of stable T cell differentiation was supported by consistent cytokine expression patterns maintained across multiple passages of selected T cell subsets. It also provided reasonable explanations why T cell responses leading to pathogen eradication would elicit similar memory responses upon antigen re-exposition. However, although theoretically intriguing, it became clear that T cell differentiation is a dynamic process with a high degree of plasticity between different T cell subsets [63, 64]. For example, Th1 cells, in certain conditions, could express cytokine profiles characteristic for Th2 cells, and Treg cells could differentiate into Th1 cells. Rather than assuming an independent intrinsic program, the microenvironment is supposed to have a major influence on T cell differentiation and function. Accordingly, pro-inflammatory T cells could be programmed tolerogenic upon entering a microenvironment that supports tolerance. With respect to the research presented in chapter 3.1, Treg differentiation will be discussed in more detail.

2.5 Regulatory T cell differentiation and function

Treg have immunosuppressive (also known as regulatory) properties and are critical for the maintenance of peripheral tolerance. They are defined CD4⁺ in combination with high expression of the IL-2 receptor α chain (CD25). Regulatory T cell function is closely associated with expression of the transcription factor forkhead box P3 (FOXP3), as mutations in this gene result in severe autoimmunity in mice and humans [58, 65-68]. Depending on their presumed developmental origin, Treg can be classified into naturally occurring (nTreg) or induced

(iTreg). nTreg are assumed to be programmed tolerogenic upon antigen encounter in the thymus (chapter 2.3), maintain their differentiation state mostly independent from the microenvironment, and play a major role in the prevention of autoimmunity [69]. iTreg on the other hand are assumed to reduce T cell function against environmental antigens [70]. However, the functional separation of nTreg and iTreg has not been completely resolved and there is evidence that suggests overlapping roles for tolerance to both self and foreign antigens [71, 72]. Independent from their individual roles, it has been shown that nTreg, which most likely correspond to CD45RA⁺ naïve Treg with consistent demethylation at the Treg-specific demethylated region (TSDR) [73-75], in contrast to CD45RA⁻ Treg, stably express FOXP3 and maintain suppressive functions mostly irrespective of exogenous cues [76]. The expression status of the IL-7 receptor (CD127) on Treg has also been shown to identify Treg with higher FOXP3 expression and possibly indicate developmental origin (nTreg vs. iTreg) [77, 78]. The exact mechanisms by which Treg induce tolerance are not entirely understood and probably diverse [79, 80].

Stable FOXP3 expression and reliable regulatory functions are critical, especially if it comes to adoptive Treg transfer. In pre-clinical and clinical settings, Treg have been expanded *in vitro* and transferred to treat autoimmune diseases [81], prevent transplant organ rejection [82], and attenuate or prevent graft versus host disease (GvHD) after allogeneic stem cell transplantation [83-85]. The exact influences of Treg phenotypes on the success of adoptive transfer have yet to be determined and are subjects of ongoing clinical trials.

2.6 T cell activation, exhaustion, and immune checkpoints

While effective T cell responses can lead to elimination of target antigens, mechanisms are required to counteract physiologic immune responses i) because the response is no longer required in case the antigen has been cleared, or ii) to prevent chronic inflammation in case the antigen cannot be cleared.

T cell-mediated immune responses include a variety of sequential processes: i) clonal selection of antigen-specific T cells by activation and proliferation in secondary lymphoid tissues, ii) trafficking to the sites of target antigen presentation, and iii) execution of inflammation and antigen elimination.

Successful T cell activation requires at least three different signals: stimulation through the TCR, amplification of TCR signaling by co-stimulatory molecules, and cytokine-mediated modulation of T cell activation and expansion. The antigen-specific part of T cell activation is mediated through TCR-peptide-MHC binding. Upon TCR activation, the interaction of co-stimulatory molecules, e.g. CD28 with CD80/CD86, strongly amplifies TCR signaling [86-90]. Cytokine secretion and signaling through cytokine receptors modulate T cell responses and support expansion [91, 92]. To counteract “over-activation”, T cells start upregulating inhibitory receptors such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and/or programmed cell death protein 1 (PD-1), among others, upon activation. Although the exact mechanisms of T cell inhibition by CTLA-4 are under debate, it has been proposed that CTLA-4 outcompetes CD80 and CD86 in binding to CD28 resulting in inhibitory signaling [93-98]. Furthermore, CTLA-4 has been shown to promote Treg functions [99, 100].

PD-1, another inhibitory molecule, physiologically limits T cell activation in the context of inflammation and autoimmunity [101-107]. Upon binding to its ligands, PD-L1 or PD-L2, PD-1 inhibits kinases that mediate T cell activation through Src homology region 2-containing protein tyrosine phosphatase 2 (SHP2) [107]. On Treg, PD-1 has been shown to enhance their proliferation [108, 109].

The transient expression of inhibitory receptors such as CTLA-4 or PD-1 is a physiologic process necessary to limit inflammation. Immune responses that lead to successful antigen eradication result in the creation of a pool of antigen-experienced memory cells that are ready to expand upon antigen re-exposure (see chapter 2.4). A key feature of the development of

functional memory compartments is that it occurs in the absence of continuing antigen stimulation [110]. In case target antigens cannot be eliminated, processes of chronic T cell activation may occur, and, in combination with lack of CD4⁺ T cell help and signals from inhibitory receptors, lead to a state called “exhaustion” [111, 112]. T cell exhaustion is characterized by loss of effector functions, upregulation and co-expression of multiple inhibitory receptors, altered expression of key transcription factors, and inability to acquire memory T cell responsiveness upon re-stimulation [113-115]. One of the critical factors for the acquisition of an exhausted T cell phenotype is chronic antigen stimulation. The longer (approx. > 1 week) the antigen stimulation the less likely is the creation of a responsive memory T cell pool [116]. Chronic antigen stimulation leads to sustained PD-1 expression via nuclear factor of activated T cells (NFAT) cytoplasmic 1 (NFATc1) providing a direct link between chronic TCR stimulation and expression of key molecules in T cell exhaustion [117]. Besides PD-1 and CTLA-4, T cells can express a variety of inhibitory receptors, such as lymphocyte activation gene 3 (LAG-3), 2B4, CD160, T cell immunoglobulin domain and mucin domain-containing protein 3 (TIM-3), among others, with presumably non-redundant functions [118]. In addition to sustained expression of inhibitory receptors, downregulation of co-stimulatory receptors such as tumor necrosis factor receptor associated factor 1 (TRAF1) contributes to exhaustion. TRAF1 has been shown to be downregulated in chronic viral infections and adoptive transfer of TRAF1⁺ cells could re-establish virus control in animal models [119]. Furthermore, CD8⁺ T cells express selected molecules that are highly associated with effector T cell function and only expressed at low levels on exhausted T cells. These markers include CD44, LY6C, killer cell lectin-like receptor subfamily G member 1 (KLRG1), and the transcription factors T-bet and EOMES [120].

Cytokines, such as IL-10, transforming growth factor (TGF)- β , and type 1 interferons (IFN- α and β) can contribute to or prevent exhaustion. It has been shown that blockade of IL-10 can,

at least in parts, restore T cell function. Type 1 interferons are critical for early virus control while chronic exposure can lead to cell death or dysfunction [121].

Of note, exhausted T cells are not considered inert but retain suboptimal functions that may or may not allow pathogen or tumor control but not elimination. Exhaustion is considered reversible as demonstrated by the clinical success of immune checkpoint blockade in reinstating immune functions especially in the field of tumor immunology [122].

Besides “exhaustion”, “anergy” and “senescence” have been defined as additional dysfunctional T cell states that cannot always be completely separated from each other. Anergy refers to an induced non-responsiveness with possible implications for peripheral tolerance. It is assumed to result from suboptimal stimulation and is characterized by low IL-2 production, low proliferative capacity, and none to low effector functions. Anergic T cells are characterized by co-expression of a variety of inhibitory receptors including LAG-3, PD-1, and CTLA-4 [110, 123]. Senescent T cells have been shown to arise from repetitive stimulation and are characterized by shortened telomeres, low telomerase activity, low proliferative capacity but high effector function. Typical phenotypic markers of senescent T cells include CD57, KLRG1, CD160, and absence of CD28 [123].

Taken together, an individual T cell’s function is defined by TCR $\alpha\beta$ sequences and characteristic expression patterns of immune phenotype, cytokine, and transcription factor genes.

2.7 Tumor-infiltrating T cells

The prognostic impact of tumor immune cell infiltration is beyond question [124-129]. Human solid and hematopoietic tumors show variable degrees of T cell infiltration and colorectal cancer was one of the first malignancies in which tumor T cell infiltration has been shown to influence prognosis independent from classical tumor node metastasis (TNM) staging [125, 128]. Tumor-infiltrating T cells can present all features of effector, memory, exhausted,

anergic, or senescent differentiation (chapter 2.6), however, particular functions and clinical implications of individual T cell populations, especially in the tumor microenvironment, are not completely understood. T cell exhaustion seems to be critically involved in tumor-directed/induced immune tolerance, at least in selected malignancies. Interference with T cell exhaustion by immune checkpoint blockade has been pioneered in malignant melanoma [99, 130-133] and resulted in impressive clinical responses in a variety of hematopoietic, e.g. Hodgkin lymphoma [134], and solid malignancies [135-138]. However, a variety of malignancies including multiple myeloma have not shown significant responses to immune checkpoint blockade alone [139].

Given our current understanding of T cell development, thymic elimination of self-reactive T cell clones, and mechanisms of peripheral tolerance (chapters 2.3-2.5), the recognition and effective elimination of tumors would require their presentation of non-self-antigens or the availability of substantial amounts of T cells that recognize self-antigens for cancer eradication. In fact, solid and hematopoietic malignancies have been shown to harbor variable frequencies of mutation-derived neo-antigens [140], which can, at least in parts, be detected by the immune system and mount effective immune responses. Nevertheless, it is not easily predictable which possible neo-antigens are effectively presented by tumor cells and are accessible for immune responses. Moreover, there is increasing evidence that the majority of tumor-infiltrating T cells, although clonally expanded, are not neo-antigen-specific [5, 141, 142].

While the clinical success of pharmacological interference with immune checkpoints is beyond question, the underlying mechanisms are incompletely understood. Not only the relatively long timespan from the beginning of checkpoint blockade to possible clinical response, phenomena like tumor pseudo-progression [143], and expression of checkpoint molecules and their ligands by a variety of cell types other than tumor and T cells suggest mechanisms of action that are more sophisticated than “simple” dis-inhibition of tumor-specific tumor-infiltrating T cell

clones. These mechanisms have to be defined in humans where malignancies have their own immunologic and genetic context resulting in a unique microenvironment that currently cannot be reliably recapitulated in animal models.

2.8 B cell maturation and determination of specificity

The immune system relies on B and plasma cells as major sources of antibodies. The critical structure that defines a B cell, determines its developmental fate and specificity is the B cell receptor, which is unique for each B cell clone.

B cell development is a continuous process beginning in primary lymphoid organs (fetal liver, bone marrow) followed by functional maturation in secondary lymphoid organs (lymph nodes, spleen) leading to immunologic memory and antibody-producing terminally differentiated plasma cells.

The initial formation of the B cell receptor genes by V(D)J rearrangement occurs in the bone marrow. The process of somatic recombination is similar to V(D)J rearrangement in T cells (chapter 2.2) but was discovered first in B cells in 1978 [144]. Diversity of the B cell receptor genes can be introduced by three different processes: i) V(D)J recombination, ii) affinity maturation and somatic hypermutation, and iii) class switch recombination. Somatic hypermutation and class switch recombination are processes unique to B cell development and do not occur in T cells or any other cell type.

Similar to rearrangement of the TCR α and β genes, recombination of the immunoglobulin (Ig) light chain genes involves V and J segments whereas heavy chains consist of V, D, and J segments. After rearrangement of D_H to J_H (immunoglobulin heavy diversity and joining segment), variable (V_H) segments recombine with the already rearranged DJ segments. The surrogate light chain together with the rearranged heavy chain forms the pre-B cell receptor in pre-B cells to enable immediate signaling once the heavy chain locus has completed rearrangement [145]. It is assumed that immediate signaling through the pre-B cell receptor

prevents the second heavy chain allele from completing rearrangement resulting in only one productive B cell receptor heavy chain rearrangement per cell (allelic exclusion). Immediate signaling requires the presence of pre-BCR ligands. It has been shown, that pre-BCR signaling is initiated independent from V_H domains [146] and bone marrow stroma cells provide ligands, such as galectin-1 or heparan sulfate [147, 148], for successful pre-BCR crosslinking. Although the concept of allelic exclusion by BCR signaling is intriguing, there is evidence that surrogate light chain expression may not be critical for allelic exclusion [149]. Recombination of the light chain segments follows after recombination of the heavy chains [150]. Signaling through the pre-BCR leads to downregulation of surrogate light chain expression giving way for a complete BCR consisting of rearranged V_H and V_L chains on immature B cells.

Immature B cells migrate into secondary lymphoid organs (spleen, lymph nodes) for further maturation in germinal centers. Germinal centers are formed by antigen presenting, activated B cells interclonally competing for stimulating interactions with T follicular helper cells [151]. They can be formed by highly proliferative B cell clones with strong antigen affinity and/or B cell clones with lower affinity in the absence of competition [152]. Germinal centers contain dark and light zones that can be identified by morphology. The dark zone is dominated by B cells at the centroblast stage, characterized by rapid proliferation and hypermutation (see below), whereas antigen presentation and affinity-dependent selection occur with the help of T follicular helper cells and follicular dendritic cells in the light zone [153-155]. Within lymph nodes, B cells can take multiple “rounds” circulating between dark and light zone of their germinal centers resulting in the selection of maximum antigen affinity B cell clones and antibody repertoire.

B cell maturation in germinal centers is fundamentally different from T cell maturation and includes antigen-dependent clonal expansion, somatic hypermutation of V genes, affinity-driven selection, and class switch recombination. While diversity of the TCR repertoire is

mainly determined by V(D)J recombination, germinal center reactions of B cells lead to additional “tuning” of specificities after completed V(D)J recombination. Somatic hypermutation in germinal centers is a critical process that introduces mostly single nucleotide exchanges in the immunoglobulin genes with a rate approx. one million-fold higher than the spontaneous mutation rates in other genes, eventually leading to the composition of higher affinity BCR genes that result in proliferation advantage [156].

The process of an activated naïve B cell expressing IgM and IgD to switch immunoglobulin expression to IgG, IgE, or IgA is called class switching. Class switching is a deletional DNA recombination process, ensures optimal effector functions of the respective antibody, and increases the chance of antigen elimination [157]. The process critically involves B cell-specific activation-induced cytidine deaminase (AID) and is initiated in secondary lymphoid organs upon antigen encounter and co-stimulatory (cytokine) signaling [158, 159].

Different stages of B cell maturation can be identified by more or less characteristic phenotypic and molecular markers. One of the most specific and earliest phenotypic markers associated with B lineage differentiation is CD19. CD20 is expressed on mature B cells and CD79a/b is present on surface immunoglobulin positive B cells whereas CD10 is relatively selectively expressed on the surface of germinal center B cells [160]. At the molecular level, a characteristic feature acquired in germinal centers is somatic hypermutation, which, if present in sufficient amounts, confirms an individual B cell clone has gone through affinity maturation in germinal centers, and can act as molecular barcode for individual B cell clones.

2.9 Plasma cells

Plasma cells are terminally differentiated B cells and the major source of circulating soluble antibodies. Upon appropriate stimulation, B cells can increase in size and start proliferation (B cell blast). Some B cell blasts may secrete antibody (usually at a frequency < 1 % of peripheral blood leukocytes) and are referred to as plasmablasts. A plasmablast without proliferation is

called plasma cell [161]. While plasmablasts circulate in peripheral blood, plasma cells can only be detected at low frequencies ($< 0.1\%$ of leukocytes) in peripheral blood of healthy individuals. Depending on their developmental path, plasma cells can be categorized into at least two different types: i) so-called short-lived plasma cells that are assumed to develop independent from germinal centers and T cell help and show (almost) no somatic hypermutations, and ii) long-lived plasma cells that have gone through germinal center reactions. In healthy individuals, more than 90 % of plasma cells arise from germinal centers. The decision of a B cell to differentiate into a plasma cell is thought to be an affinity-driven event involving T cell help [153, 162]. Whether plasma cell differentiation occurs outside or inside the germinal center has been shown, at least in parts, to be determined by the nature of the stimulating antigen [163, 164]. T cell independent antigens such as Toll-like receptor ligands (LPS, TLR4 agonist) including polysaccharides from bacterial cell walls can stimulate B cells in addition to BCR aggregation and induce the development of short-lived plasma cells. Their survival has been shown to be dependent on B-cell activating factor belonging to the tumor necrosis family (BAFF) [165].

Plasma cells are phenotypically and morphologically distinct from B cells and plasmablasts allowing their reliable detection by microscopy and flow cytometry. In contrast to mature B cells, plasma cells are usually negative for CD20 and surface immunoglobulin. They can be identified by the uniquely high expression of CD38 and CD138. Long- and short-lived plasma cells cannot be reliably distinguished by phenotype but by numbers of somatic hypermutations determined with sequencing. As long-lived plasma cells originate from germinal centers, they usually harbor substantial amounts of somatic hypermutations.

2.10 Multiple Myeloma

Multiple myeloma is characterized by the accumulation of monoclonal plasma cells in the bone marrow and most if not all cases develop from a non-malignant pre-cancer called monoclonal

gammopathy of undetermined significance (MGUS). MGUS is defined by the presence of monoclonal immunoglobulin and lack of symptoms in often undiagnosed patients. As outlined in chapter 2.9, non-malignant plasma cells usually show no or at most very low proliferative activity. In multiple myeloma, (epi-)genetic aberrations are assumed to restore proliferative capacity in variable proportions of plasma cells, which correlates with prognosis and enables malignant clonal expansion [166].

Clinically, multiple myeloma frequently presents with secondary organ damage such as renal failure, anemia, thrombocytopenia, increased frequencies of infections, insufficiency fractures, pain due to lytic bone lesions and/or tissue mass, and thrombotic/thromboembolic events among others. Multiple myeloma is diagnosed by histology, flow cytometry, and (molecular) genetics of a bone marrow sample in the context of clinical features. While thrombocytopenia and anemia can be due to high degrees of bone marrow plasma cell infiltration displacing erythropoiesis, many manifestations are not directly related to the malignant plasma cells.

Multiple myeloma is unique in programming its microenvironment to support tumor growth [167], protect from tumor-directed T cell responses [168] and chemotherapeutics [169-171]. Almost all sorts of immune cells including T-, B-, NK-, and dendritic cell compartments show diseases-associated features [172, 173]. Bone destruction can be mediated by direct interactions of myeloma cells with osteocytes, which support myeloma cell survival and promote the activation of osteoclasts, leading to continuous bone destruction [174].

Microenvironmental features in combination with (epi-)genetic aberrations [175-179] within the multiple myeloma cells themselves result in intra- and interclonal heterogeneity of the malignant plasma cells. Heterogeneity can be detected by morphology, expression of surface markers, (epi-)genetics, and gene expression, among others. Despite phenotypic heterogeneity, all malignant cells share identical immunoglobulin light and heavy chain gene sequences, which can act as molecular barcodes to track multiple myeloma cells at the single cell level.

The substantial number of somatic hypermutations and completed class switch recombination suggest that the disease originates from the (post) germinal center stage of B cell development [180-182].

The phenotypic range of multiple myeloma cells and whether the malignant clones include normal-phenotype (memory) B lineage cells have been matters of debate [183-195]. However, as outlined above, immune phenotypes of molecularly defined clones can only be accurately studied at the single cell level. To contribute to the definition of the phenotypic range that individual multiple myeloma clones can occupy, this work introduces technologies to track clone-associated immune phenotypes at the single cell level.

Considering the broad effects on the bone marrow microenvironment, the compartment where hematopoiesis takes place, changes in immune cell phenotypes detectable with high-dimensional technologies could be assumed, possibly allowing the distinction of multiple myeloma, MGUS, and healthy individuals based on peripheral blood immune signatures. Previous studies on peripheral blood immune phenotypes of multiple myeloma patients were limited with regard to analysis depth, which could be substantially increased by the application of CyTOF that uses metal-labeled antibodies for immune phenotyping in the presented research. Allowing the simultaneous detection of more than 30 markers on millions of single cells, CyTOF outcompetes FACS in analysis depth suggesting that with this technology immune phenotype differences between disease groups might be detectable in high-dimensional space.

2.11 Rectal cancer

Colorectal cancer ranks among the four most frequent cancer entities and the five year survival rate is approximately 64 % for all stages combined [196]. In 95 % of all cases, colorectal cancer histologically presents as adenocarcinoma, rare cases are carcinoids or sarcomas. The disease

is classified according to the TNM system and staged according to the Union for International Cancer Control (UICC) [197]. Similar to multiple myeloma, which develops from a pre-cancer stage, colorectal cancer is a prime example for cancer developing from non-cancerous pre-malignant lesions. It is generally accepted that most cases of colorectal cancer are preceded by adenoma. The adenoma-carcinoma sequence is a longstanding process (except in selected genetically driven, possibly inherited cases) associated with accumulating acquisition of genetic aberrations [198]. Along with the (usually slow) progression from adenoma to carcinoma, (pre-)cancerous lesions are assumed to induce specialized microenvironments including attraction and re-programming of immune cells. Colorectal cancer was one of the first malignancies in which the pivotal role of immune cell infiltration for cancer control could be demonstrated. Especially type and density of tumor-infiltrating T cells have been shown to influence survival independent from classical TNM staging [124, 127-129, 199, 200]. However, functions and targets of tumor-infiltrating immune cells remain largely unknown. An attractive concept of T cell tumor control is that tumor-infiltrating T cells (TILs) recognize and kill tumor cells based on their presentation of mutation-derived neo-antigens. In fact, TILs have been shown to recognize neo-antigens [201] and tumors with high mutational load were especially responsive to presumably T cell-dependent therapeutics (immune checkpoint blockade) [202, 203].

Prognosis and clinical course in colorectal cancer do not only depend on immune cell infiltration but also on the location of the tumor. Carcinomas located at the proximal or distal part of the colon differ significantly with regard to histology and genetic features. While proximal carcinomas are more frequently mucinous and microsatellite instable, distal carcinomas are more often epidermal growth factor receptor (EGFR) or human epidermal growth factor receptor 2 (HER2) amplified, associated with poor survival after relapse [204, 205].

The work presented in chapter 3.4 is particularly focused on TIL immune phenotypes and functions associated with clonal – possibly tumor-specific – expansion in comparison with T cells from unaffected mucosa and peripheral blood.

The following selected publications introduce methodologies for in-depth determination of phenotypes, differentiation states, functions, and clonal relatedness of T and B cells in bulk and at the single cell level. These technologies were applied to

- i) identify differentiation fate of regulatory T cells upon loss of FOXP3 expression during *in vitro* expansion in the setting of allogeneic stem cell transplantation,
- ii) identify clonal expansion, functions, and distribution of target antigens of clonally expanded rectal cancer-infiltrating T cells at the single cell level, and
- iii) phenotypically track selected B lineage clones in multiple myeloma bone marrow at the single cell level

Major innovations include (1) the development of highly efficient high-throughput single cell methodologies for detailed phenotyping, functional assessment, and molecular tracking of individual cells, (2) the focus on human malignancies and human tumor immunology entirely studied in human subjects, leading to (3) insights into rectal cancer and multiple myeloma immunology that have impact on our understanding of disease pathogenesis and T cell infiltration with possible consequences for novel (immuno-)therapies.

2.12 Scientific questions

The presented studies are focused on human T and B cell biology in rectal cancer and multiple myeloma. Both malignancies represent prime examples of tumor-microenvironment interactions and tumor-infiltrating lymphocytes have been shown to influence prognosis independent from classical staging.

Chapter 3 presents the development of novel high-dimensional single cell methodologies that were applied to answer the following research questions:

- Are malignancy-associated immune phenotypes as measures of disease activity detectable in peripheral blood and within the tumor microenvironment?
- What is the phenotypic range of multiple myeloma?
- Is clonal expansion of tumor-infiltrating T cells restricted to certain immune phenotypes, compartments, and target peptide specificities?

The studies aim at a better understanding of human disease biology, possible definition of cellular therapeutic targets, and identification of biological specimens most likely containing malignancy-targeting immune cells.

3. Own original research (Eigene Arbeiten)

This work is focused on regulation of tolerance and tumor-directed immune responses in solid and hematopoietic malignancies. The following research will

- i) provide methodologies for gene expression analysis of regulatory T cells leading to the definition of their developmental fate upon loss of regulatory functions.
- ii) introduce high-throughput methodologies for the determination of T cell specificity, immune phenotype, cytokine, and transcription factor expression at the single cell level.
- iii) determine the spatial distribution, functional profile, and accessibility of clonally expanded tumor-infiltrating T cells in rectal cancer.
- iv) apply CyTOF – one of the most powerful technologies for high-throughput immune phenotyping at the protein and single cell level – to identify a novel, expanded B cell phenotype in peripheral blood of patients with active multiple myeloma.
- v) determine the phenotypic range of multiple myeloma B lineage cells applying a combination of multi-parameter FACS single cell index sorting and immunoglobulin light chain sequencing.

3.1 Transcription factor-specific cell sorting for gene expression analyses

T cell differentiation states have been defined by characteristic patterns of surface molecule, cytokine and transcription factor expression. They can be affected by various cues resulting in T cell plasticity. As an example, Treg, which are T helper cells characterized by high expression of the IL-2 receptor α chain, the transcription factor FOXP3, and cytokine profiles associated with immune tolerance, can lose regulatory properties and drive inflammation. T cell plasticity is especially important in the setting of adoptive cell transfer where cell products with precisely defined specificities and functions are required. Treg can be adoptively

transferred in various clinical settings including prevention of GvHD after allogeneic stem cell transplantation. Due to their low abundance, Treg have to be *in vitro* expanded before adoptive transfer and subsets of Treg lose characteristic FOXP3 expression during this process. The following publication determines the fate of Treg that lose regulatory properties by specifically sorting Treg that maintained or downregulated FOXP3 expression after *in vitro* expansion for subsequent RNA extraction and whole genome microarray analysis. This approach is particularly challenging as intranuclear FOXP3 staining requires fixation and permeabilization, which makes DNA and RNA inaccessible after conventional staining procedures.

Publication 1

Hansmann L, Schmidl C, Kett J, Steger L, Andreesen R, Hoffmann P, Rehli M, Edinger M. Dominant Th2 differentiation of human regulatory T cells upon loss of FOXP3 expression. J Immunol. 2012; 188:1275-82. DOI: <https://doi.org/10.4049/jimmunol.1102288>

Abstract

“CD4⁺CD25⁺FOXP3⁺ regulatory T cells (Treg) are pivotal for peripheral self-tolerance. They prevent immune responses to auto- and alloantigens and are thus under close scrutiny as cellular therapeutics for autoimmune diseases and the prevention or treatment of alloresponses after organ or stem cell transplantation. We previously showed that human Treg with a memory cell phenotype, but not those with a naive phenotype, rapidly downregulate expression of the lineage-defining transcription factor FOXP3 upon *in vitro* expansion. We now compared the transcriptomes of stable FOXP3⁺ Treg and converted FOXP3⁻ ex-Treg by applying a newly developed intranuclear staining protocol that permits the isolation of intact mRNA from fixed, permeabilized, and FACS-purified cell populations. Whole-genome microarray analysis revealed strong and selective upregulation of Th2 signature genes, including GATA-3, IL-4, IL-5, and IL-13, upon downregulation of FOXP3. Th2 differentiation

of converted FOXP3⁻ ex-Treg occurred even under nonpolarizing conditions and could not be prevented by IL-4 signaling blockade. Thus, our studies identify Th2 differentiation as the default developmental program of human Treg after downregulation of FOXP3.”

Publication 1

Hansmann L, Schmidl C, Kett J, Steger L, Andreesen R, Hoffmann P, Rehli M, Edinger M.
Dominant Th2 differentiation of human regulatory T cells upon loss of FOXP3 expression. J
Immunol. 2012; 188:1275-82. DOI: <https://doi.org/10.4049/jimmunol.1102288>

3.2 Determination of T cell differentiation and specificity at the single cell level

Although intranuclear staining and FACS sorting of bulk populations with subsequent RNA extraction allowed the identification of Th2 differentiation as the default differentiation of Treg upon loss of FOXP3 expression during *in vitro* expansion, the approach had substantial shortcomings: i) Th2 could be identified as dominant differentiation pathway, however, the data suggested a mixture of various differentiation states, which could not be clearly assigned to individual cells or populations. ii) Due to technical reasons, our protocol did not allow FACS sorting based on cytokine expression, which is key for the accurate determination of T cell function. iii) T cell specificity (TCR sequence) along with cytokine and transcription factor profiles cannot be determined in bulk populations and require single cell resolution.

The following publication presents a deep sequencing-based methodology for high-throughput determination of paired TCR $\alpha\beta$ sequences, cytokine, and transcription factor expression in single cells. Paired TCR $\alpha\beta$ sequencing allows reconstruction and expression of single TCRs of choice for functional assays and determination of specificity (chapter 3.4).

Publication 2

Han A, Glanville J, Hansmann L, Davis MM. Linking T-cell receptor sequence to functional phenotype at the single-cell level. *Nat Biotechnol.* 2014; 32:684-92. DOI: <https://doi.org/10.1038/nbt.2938>

Abstract

“Although each T lymphocyte expresses a T-cell receptor (TCR) that recognizes cognate antigen and controls T-cell activation, different T cells bearing the same TCR can be functionally distinct. Each TCR is a heterodimer, and both alpha- and beta-chains contribute to determining TCR antigen specificity. Here we present a methodology enabling integration of information about TCR specificity with information about T cell function. This method involves sequencing of TCRalpha and

TCRbeta genes, and amplifying functional genes characteristic of different T cell subsets, in single T cells. Because this approach retains information about individual TCRalpha-TCRbeta pairs, TCRs of interest can be expressed and used in functional studies, for antigen discovery, or in therapeutic applications. We apply this approach to study the clonal ancestry and differentiation of T lymphocytes infiltrating a human colorectal carcinoma.”

Publication 2

Han A, Glanville J, Hansmann L, Davis MM. Linking T-cell receptor sequence to functional phenotype at the single-cell level. Nat Biotechnol. 2014; 32:684-92. DOI: <https://doi.org/10.1038/nbt.2938>

3.3 Combination single cell sequencing with high-dimensional FACS index sorting

Single cell TCR $\alpha\beta$ and phenotype sequencing enables accurate determination of phenotypes by gene expression analysis with a gene-targeted sequencing panel resulting in binary (expressed/not expressed) data for each particular cell and gene of interest. Some markers, especially those associated with activation, exhaustion, senescence, and other functional states, show function-associated continuous expression and may be affected by post-transcriptional regulation, requiring determination at the protein level. E.g. high CD25 expression on CD4⁺ T cells is associated with suppressive functions of Treg while intermediate CD25 expression can result from activation and is associated with a variety of different functions.

In the following study, the single cell sequencing protocol presented in chapter 3.2 was expanded with multi-parameter FACS index-sorting. Index-sorting records fluorescence and scatter characteristics for each single sorted cell so they can be read out retrospectively combining the analytical power and accuracy of flow cytometry at the protein level with downstream single cell sequencing.

Publication 3

Penter L, Dietze K, Bullinger L, Westermann J, Rahn HP, Hansmann L. FACS single cell index sorting is highly reliable and determines immune phenotypes of clonally expanded T cells. Eur J Immunol. 2018; 48:1248-50. DOI: <https://doi.org/10.1002/eji.201847507>

Abstract

“FACS index sorting allows the isolation of single cells with retrospective identification of each single cell's high-dimensional immune phenotype. We experimentally determine the error rate of index sorting and combine the technology with T cell receptor sequencing to identify clonal T cell expansion in aplastic anemia bone marrow as an example.”

Publication 3

Penter L, Dietze K, Bullinger L, Westermann J, Rahn HP, Hansmann L. FACS single cell index sorting is highly reliable and determines immune phenotypes of clonally expanded T cells. Eur J Immunol. 2018; 48:1248-50. DOI: <https://doi.org/10.1002/eji.201847507>

3.4 Definition of immune phenotypes and target antigen distribution of clonally expanded, selectively rectal cancer-infiltrating T cells

The work presented in chapters 3.1-3.3 built the methodological foundation to determine T cell differentiation, functions and specificities at the single cell level in rectal cancer in the following study.

Publication 4

Penter L, Dietze K, Ritter J, Lammoglia-Cobo MF, Garmshausen J, Aigner F, Bullinger L, Hackstein H, Wienzek-Lischka S, Blankenstein T, Hummel M, Dornmair K, Hansmann L. Localization-associated immune phenotypes of clonally expanded tumor-infiltrating T cells and distribution of their target antigens in rectal cancer. *OncoImmunology* 2019. DOI: <https://doi.org/10.1080/2162402X.2019.1586409>

Abstract

“The degree and type of T cell infiltration influence rectal cancer prognosis regardless of classical tumor staging. We asked whether clonal expansion and tumor infiltration are restricted to selected-phenotype T cells; which clones are accessible in peripheral blood; and what the spatial distribution of their target antigens is.

From five rectal cancer patients, we isolated paired tumor-infiltrating T cells (TILs) and T cells from unaffected rectum mucosa (T_{UM}) using 13-parameter FACS single cell index sorting. TCR $\alpha\beta$ sequences, cytokine, and transcription factor expression were determined with single cell sequencing.

TILs and T_{UM} occupied distinct phenotype compartments and clonal expansion predominantly occurred within CD8⁺ T cells. Expanded TIL clones identified by paired TCR $\alpha\beta$ sequencing and exclusively detectable in the tumor showed characteristic PD-1 and TIM-3 expression. TCR β repertoire sequencing identified 49 out of 149 expanded TIL clones circulating in peripheral blood and 41 (84 %) of these were PD-1⁺ TIM-3⁺.

To determine whether clonal expansion of predominantly tumor-infiltrating T cell clones was driven by antigens uniquely presented in tumor tissue, selected TCRs were reconstructed and incubated with cells isolated from corresponding tumor or unaffected mucosa. The majority of clones exclusively detected in the tumor recognized antigen at both sites.

In summary, rectal cancer is infiltrated with expanded distinct-phenotype T cell clones that either i) predominantly infiltrate the tumor, ii) predominantly infiltrate the unaffected mucosa, or iii) overlap between tumor, unaffected mucosa, and peripheral blood. However, the target antigens of predominantly tumor-infiltrating TIL clones do not appear to be restricted to tumor tissue.”

Publication 4

Penter L, Dietze K, Ritter J, Lammoglia-Cobo MF, Garmshausen J, Aigner F, Bullinger L, Hackstein H, Wienzek-Lischka S, Blankenstein T, Hummel M, Dornmair K, Hansmann L. Localization-associated immune phenotypes of clonally expanded tumor-infiltrating T cells and distribution of their target antigens in rectal cancer. OncoImmunology 2019. DOI: <https://doi.org/10.1080/2162402X.2019.1586409>

3.5 Mass cytometry and single cell immunoglobulin sequencing identify a novel memory B cell subset in patients with active multiple myeloma

FACS single cell index sorting and targeted gene expression sequencing are limited with respect to numbers of parameters analyzed in parallel. A particular challenge relied in applying high-dimensional phenotyping approaches to hematopoietic malignancies aiming at the identification of disease-associated immune patterns in peripheral blood. In the following study, multiple myeloma, which is defined by the accumulation of monoclonal plasma cells in the bone marrow, was chosen as an example. The disease develops from a non-malignant precursor called monoclonal gammopathy of undetermined significance (MGUS) and the study hypothesized to identify immune phenotypes that distinguished healthy individuals, MGUS patients, and multiple myeloma patients. Multiple myeloma plasma cells are almost exclusively located in the bone marrow, however, bone marrow samples, especially from MGUS patients or healthy individuals, are not easily accessible. The disease is known for its substantial effects on the bone marrow microenvironment – the compartment where hematopoiesis takes place. Therefore, the identification of disease state-associated phenotypes even in the peripheral blood - if determined at sufficient depth – could be assumed. Disease-associated phenotypes were not expected to be restricted to T cell compartments but the study proposed a broad view at the entire cellular immune system. Therefore, one particular challenge was the most accurate determination of immune phenotypes in highest possible depth at the single cell level. Single cell phenotyping with conventional flow cytometry is usually focused on particular immune cell compartments and limited to approximately 12-15 markers due to spectral overlap of fluorochrome-labeled antibodies. Peripheral blood contains numerous cellular and non-cellular components with hundreds to thousands of individual phenotypes limiting the chances of success to detect multiple myeloma-associated immune phenotypes by conventional flow cytometry.

In the following work, CyTOF, which allows simultaneous detection of more than 30 metal-labeled antibodies at the single cell level, identified a novel CD20⁺CD38⁺CD24^{lo}CD27⁺ peripheral blood B cell subset that was specifically expanded in patients with active multiple myeloma.

Publication 5

Hansmann L, Blum L, Hsin-Ju C, Liedtke M, Robinson W, Davis MM. Mass cytometry analysis shows that a novel memory phenotype B cell is expanded in multiple myeloma. *Cancer Immunol Res.* 2015; 3:650-60. DOI: <https://doi.org/10.1158/2326-6066.CIR-14-0236-T>

Abstract

“It would be very beneficial if the status of cancers could be determined from a blood specimen. But peripheral blood leukocytes are very heterogeneous between individuals and thus high resolution technologies are likely required. We used Cytometry by Time of Flight (CyTOF) and next generation sequencing to ask whether a plasma cell cancer (multiple myeloma) and related pre-cancerous states had any consistent effect on the peripheral blood mononuclear cell phenotypes of patients. The analysis of 13 cancer patients, 9 pre-cancer patients, and 9 healthy individuals revealed significant differences in the frequencies of the T, B, and natural killer cell compartments. Most strikingly, we identified a novel B cell population that normally accounts for 4.0+/-0.7% (mean+/-SD) of total B cells and is up to 13-fold expanded in multiple myeloma patients with active disease. This population expressed markers previously associated with both memory (CD27⁺) and naive (CD24^{lo}CD38⁺) phenotypes. Single cell immunoglobulin gene sequencing showed polyclonality, indicating that these cells are not precursors to the myeloma, and somatic mutations, a characteristic of memory cells. SYK, ERK, and p38 phosphorylation responses, and the fact that most of these cells

expressed isotypes other than IgM or IgD, confirmed the memory character of this population, defining it as a novel type of memory B cells.”

Publication 5

Hansmann L, Blum L, Hsin-Ju C, Liedtke M, Robinson W, Davis MM. Mass cytometry analysis shows that a novel memory phenotype B cell is expanded in multiple myeloma. *Cancer Immunol Res.* 2015; 3:650-60. DOI: <https://doi.org/10.1158/2326-6066.CIR-14-0236-T>

3.6 Definition of the phenotypic range of multiple myeloma B lineage cells

Although CD38⁺CD24^{lo}CD27⁺ expanded memory B cells in multiple myeloma were unlikely to be part of malignant B lineage expansion, the phenotypic range an individual multiple myeloma clone can occupy remained as an unanswered question. As already demonstrated in T cells, phenotypes and clonal relationships can only be reliably determined at the single cell level.

The following publication presents a methodology that combined multi-parameter FACS index sorting with high-throughput single cell immunoglobulin light chain sequencing to determine the phenotypic range which individual B cell clones can occupy in multiple myeloma bone marrow.

Publication 6

Hansmann L, Han A, Penter L, Liedtke M, Davis MM. Clonal Expansion and Interrelatedness of Distinct B-Lineage Compartments in Multiple Myeloma Bone Marrow. *Cancer Immunol Res.* 2017; 5:744-54. DOI: <https://doi.org/10.1158/2326-6066.CIR-17-0012>

Abstract

“Multiple myeloma is characterized by the clonal expansion of malignant plasma cells in the bone marrow. But the phenotypic diversity and the contribution of less predominant B-lineage clones to the biology of this disease have been controversial. Here, we asked whether cells bearing the dominant multiple myeloma immunoglobulin rearrangement occupy phenotypic compartments other than that of plasma cells. To accomplish this, we combined 13-parameter FACS index sorting and t-Stochastic Neighbor Embedding (t-SNE) visualization with high-throughput single-cell immunoglobulin sequencing to track selected B-lineage clones across different stages of human B-cell development. As expected, the predominant clones preferentially mapped to aberrant plasma cell compartments, albeit phenotypically altered from wild

type. Interestingly, up to 1.2% of cells of the predominant clones colocalized with B-lineage cells of a normal phenotype. In addition, minor clones with distinct immunoglobulin sequences were detected in up to 9% of sequenced cells, but only 2 out of 12 of these clones showed aberrant immune phenotypes. The majority of these minor clones showed intraclonal silent nucleotide differences within the CDR3s and varying frequencies of somatic mutations in the immunoglobulin genes. Therefore, the phenotypic range of multiple myeloma cells in the bone marrow is not confined to aberrant-phenotype plasma cells but extends to low frequencies of normal-phenotype B cells, in line with the recently reported success of B cell-targeting cellular therapies in some patients. The majority of minor clones result from parallel nonmalignant expansion.”

Publication 6

Hansmann L, Han A, Penter L, Liedtke M, Davis MM. Clonal Expansion and Interrelatedness of Distinct B-Lineage Compartments in Multiple Myeloma Bone Marrow. *Cancer Immunol Res.* 2017; 5:744-54. DOI: <https://doi.org/10.1158/2326-6066.CIR-17-0012>

4. Discussion (Diskussion)

This work presents an approach to human immunology in hematopoietic and solid malignancies. A major challenge relies in the heterogeneity of human study populations. “Heterogeneity” refers to the fact that diversity in age and gender, genetic variation, history of other diseases, medications, accumulating exposure to mutagens and/or toxins, previous immune challenges possibly associated with infectious diseases, vaccination, and many others lead to an almost infinite diversity of immune patterns, which, in their entire complexity, we are currently unable to understand. Several attempts have been made to reduce heterogeneity by studying cancer immunology in more or less homogenous animal models. While animal models represent the environments of choice to study basic gene and protein function *in vivo*, there is substantial evidence from the last decades that human immunology in the context of cancer can be most reliably studied in humans [206-209]. Novel technologies such as deep sequencing and a variety of multi-dimensional high-throughput single cell technologies have revolutionized the (human) immunology field allowing analyses in unprecedented depth at the single cell level.

This work (chapter 3) presents methodologies for the determination of lymphocyte specificity in combination with high-dimensional surface and intracellular marker, transcription factor, and cytokine expression of bulk cells and at the single cell level. These methodologies were combined with CyTOF phenotyping and TCR reconstruction in reporter cell lines to determine Treg plasticity during *in vitro* expansion and track individual T and B lineage clones in rectal cancer and multiple myeloma. The identification of a novel, expanded B cell subset in multiple myeloma, a disease that has already been extensively studied during the last decades, underlines the power of these high-dimensional approaches. The presented findings have significant impact on cellular and cancer immunology therapeutics and, entirely derived from human subjects, have high and immediate translational potential.

4.1 Regulatory T cell plasticity and cellular therapy

Treg have been in high demand for adoptive therapy of autoimmune diseases and in the setting of solid organ or stem cell transplantation to prevent organ rejection and GvHD. The bottleneck for adoptive Treg transfer is their low abundance (1 % - 10 % of T helper cells) in peripheral blood requiring *in vitro* expansion.

T helper cell subsets, especially Treg, have been defined by their expression of lineage-defining transcription factors [58-61] and Treg function has been shown to be critically dependent on FOXP3 expression [210]. Currently, a variety of different protocols for *in vitro* expansion of regulatory T cells find application in (pre-)clinical trials and animal models in transplantation and treatment/prophylaxis of autoimmunity [83-85, 211-215]. Depending on Treg population characteristics and expansion conditions (supplements of cytokines or antibiotics/immunosuppressants), substantial proportions of Treg have been shown to lose FOXP3 expression during the expansion process.

Methodologies presented in chapter 3.1 [1] enable the isolation of intact RNA after fixation and permeabilization of plasma and nuclear membranes, which is critical for transcription factor staining beyond FOXP3 and makes transcription factor-sorted cell populations accessible for reliable RNA-based downstream analyses. Possibly due to different mechanisms of fixation (ethanol vs. paraformaldehyde), the protocol does not allow intracellular cytokine staining, which seems to require paraformaldehyde fixation.

It has been shown that especially the CD45RA⁻ subpopulation of Treg loses FOXP3 expression during *in vitro* expansion [76, 216] accompanied by epigenetic modifications involving the FOXP3 promoter and TSDR [75, 217-219]. Nevertheless, this population has not been excluded from cell products in a variety of clinical trials. The data in chapter 3.1 demonstrate that under non-polarizing conditions FOXP3⁻ ex-Treg predominantly acquire Th2 phenotypes [1]. This finding is critical in the setting of adoptive T cell transfer where cell products are

required to maintain defined differentiation states and functions. The results do not allow prediction which differentiation paths will be followed *in vivo* but suggest and confirm that plasticity among T helper cells does include almost all T helper differentiation states and is more pronounced than was initially assumed.

The determination and definition of T cell differentiation is further complicated by (potentially stimulation-induced) changes of individual cells' cytokine and transcription factor profiles over time. It has been shown that the magnitude and type of cytokines secreted by T cells follow distinct patterns after stimulation [220-222]. The analysis of cells after fixation can only capture single time points, however, strategies profiling thousands of single cells in parallel in multi-dimensional space have proven T cell differentiation is a dynamic process including phenotypic changes and overlapping transitional states [223].

To which extent T cell plasticity impacts the desired effects of adoptively transferred T cell preparations can eventually be deduced from ongoing clinical trials. In case of adoptive transfer of tumor antigen-specific T cells for cancer treatment, it has already been shown that immune phenotype changes occurring *in vivo* after cell transfer can interfere with anti-tumor effects [224, 225].

4.2 Lymphocyte function and specificity at single cell resolution

Antigen specificity of T or B cells is encoded in the TCR $\alpha\beta$ or immunoglobulin heavy and light chain genes respectively (chapters 2.2 and 2.8). As almost unlimited diversity of TCR and immunoglobulin genes in combination with a variety of cellular functions can be expected, the accurate parallel determination of specificity and function requires single cell resolution. This work presents the development of methodologies that combine FACS single cell index sorting with gene-targeted PCR amplification of TCR, immunoglobulin light chain, and selected phenotype genes for subsequent single cell sequencing. Index sorting is a modification of conventional FACS sorting, which records high-dimensional fluorescence and scatter

information for each single sorted cell so they can be read out retrospectively. This approach can link phenotype data at the protein level to downstream, in this case single cell sequencing, information.

The protocols presented in chapter 3.2 were published at a time when other laboratories established approaches for high throughput single cell RNA sequencing (RNAseq), which promise quantitative accessibility of the whole transcriptome in single cells [226-228]. Both technologies have their advantages and disadvantages. Our methodologies allow the determination of paired TCR $\alpha\beta$ sequences in > 90 % and immunoglobulin light chain sequences in > 70 % of sorted cells. The combination with FACS index sorting results in an accuracy of the assigned immune phenotypes in > 99 % of all sorted cells [3], albeit with a previously defined, limited panel of parameters. Single cell RNAseq approaches result in a gene coverage of 10-30 % per cell [227] and are limited regarding the reconstruction of entire TCR $\alpha\beta$ and immunoglobulin sequences. The strength of high-throughput single cell RNAseq relies in the analysis of thousands of cells and markers in parallel possibly leading to the discovery of novel cell populations and population characteristics. However, our technologies presented in chapters 3.2 and 3.3 outperform all currently available single cell RNAseq technologies with regard to sequencing efficiency, phenotype accuracy, and represent the approach of choice if reconstruction of TCR or immunoglobulin genes is desired. The unique power is confirmed by other groups adapting and developing these methodologies in particular for TCR sequence determination and reconstruction in times when single cell RNAseq approaches have become commercially available at affordable cost [229]. In conclusion, the presented methodologies and single cell RNAseq have to be considered complementary and should be carefully chosen depending on the research questions.

4.3 Identification of ligands of TCRs with unknown specificities

Although technologies for sequencing TCR and immunoglobulin genes have become increasingly available, there is currently no straight forward methodology for the identification of unknown TCR ligands. T cells recognize their target peptides in complex with MHC class I or II (chapter 2.1). In the 1990-ies, John Altman and Mark Davis developed peptide-MHC tetramers to label T cells with common specificities for a selected target peptide [230]. This landmark technology allows the identification of T cells specific for virtually any peptide, however, the exact peptide sequence and MHC-restriction have to be known. For example, peptide-MHC tetramers can identify T cells that recognize selected Epstein-Barr virus antigens with high sensitivity and specificity. Vice versa, the identification of the target peptide of an individual TCR is more complex. Even if a particular TCR sequence is known, the exact structure and interaction with the target peptide-MHC are almost impossible to predict. Bioinformatically, sophisticated algorithms for grouping TCRs by presumed common specificities and attempts to read potential ligands from TCR sequences have only been partially successful and rely on the inclusion of reference TCRs with known specificities [231, 232].

Experimentally, there have been a few approaches for the unbiased identification of unknown TCR ligands that rely on recombinant expression of the TCR of interest and presentation of plasmid-encoded highly diverse peptide libraries on HLA-recombinant antigen presenting cells [233, 234]. These technologies are labor-intensive and lead in the best-case scenario to a selection of candidate peptides. The identification of the target protein requires further time-consuming experimentation and is limited by methodologies that require processing and antigen presentation of the presumed target proteins, which can be substantially different *in vitro* compared to *in vivo* situations.

The presented research (chapter 3.4) determined whether particular TCRs recognized target antigen in leftover cells from rectal cancer tissues [5]. The application of TCR recombinant reporter cell lines [234] demonstrated that target antigens of T cell clones uniquely expanded in the rectal cancer microenvironment were not restricted to tumor tissue [5]. These findings support recent studies suggesting that the majority of tumor-infiltrating T cells are not specific for mutation-derived neo-antigens but maybe not directly tumor-related so-called bystander T cells [142, 235]. The presented data on distribution of target antigens of clonally expanded TILs and their accessibility in peripheral blood question our current understanding of the roles of TILs and mechanistic effects of immune checkpoint blockade.

4.4 Technologies for high-dimensional phenotyping – CyTOF and beyond

In the study presented in chapter 3.5, CyTOF could identify multiple myeloma-associated immune phenotypes in peripheral blood [6]. Multiple myeloma is a prime example for tumor-microenvironment interactions [170], leading to various effects on the bone marrow – the compartment where hematopoiesis takes place. Besides conventional somatic mutations in genes such as KRAS, NRAS, among others, mutated immunoglobulin genes can be presented as neo-antigens possibly leading to measurable differences in the immune cell compartments of multiple myeloma, MGUS patients, and healthy individuals. The presented approach (chapter 3.5) was particularly focused on differences in T and B cell compartments but did not exclude NK cells, monocytes, and dendritic cells. With respect to monocytes, dendritic cells and their responses to TLR ligand or Phorbol Myristate Acetate (PMA)/Ionomycin stimulation, availability of freshly isolated peripheral blood mononuclear cells (PBMCs) without prior cryopreservation was critical. The B cell compartment, in case of multiple myeloma and MGUS, is part of the malignancy and therefore the detection of differences between patients and healthy individuals could be expected. Given the complexity of possible variation of immune phenotypes and functions between multiple myeloma, MGUS, and healthy individuals

in an inherently heterogeneous human population, a systems immunology approach was likely required. Conventional flow cytometry, which is limited to 12-15 markers depending on cytometer setup and researcher experience, cannot resolve changes in various immune cell compartments simultaneously at sufficient depth; CyTOF can measure 38 and more parameters in parallel [6]. The identification of a novel B cell subset in a malignancy that has been extensively characterized by conventional flow cytometry in the last decades underlines the analytical power of this approach.

Although substantially expanding the analytical depth, CyTOF is limited by the accessibility of commercially available metals for antibody labeling, lack of straight-forward data analysis tools, restricted accessibility of the technology, and relatively time-consuming training compared to conventional flow cytometry. Furthermore, mass cytometry ultimately destroys the cells making them inaccessible for downstream analyses such as TCR or immunoglobulin sequencing.

Recent developments including nucleic acid oligo-tagged antibodies in combination with single cell next generation sequencing for antibody detection promise phenotypic and genetic characterization of single cells in virtually unlimited depth [236, 237]. Currently, these technologies are limited with regard to intracellular staining and sequencing efficiency but can be expected to substantially improve.

In conclusion, FACS is the technology of choice for high-dimensional phenotyping at the single cell and protein level if cells need to be available for downstream applications. CyTOF outperforms conventional flow cytometry in analysis depth and ultimately destroys the cells. Nucleic acid oligo-labeled antibodies promise a substantial increase in analysis depth in combination with single cell transcriptomics. If the technology can live up to its promises will become clear in the next years.

4.5 Implications of the presented findings in rectal cancer

Colorectal cancer is a prime example for the prognostic significance of tumor-infiltrating T cells. The disease biology is heterogeneous depending on the (epi-)genetic background but also on the location of the tumor within the colo-rectum [204, 205]. To minimize location-associated immune phenotype differences, the study population in chapter 3.4 was restricted to rectal cancer patients.

The tumor microenvironment contributes to disease biology and is expected tolerogenic in rectal cancer. Data in chapter 3.4 support the induction of T cell tolerance by i) expression of checkpoint molecules on clonally expanded, and ii) expression of FOXP3 in non-expanded TILs. Furthermore, the data suggest, at least for the included checkpoint molecules, that tolerance in clonally expanded T cells is most likely not dependent on one single checkpoint molecule (e.g. PD-1) but sustained by complex expression patterns of various checkpoint molecules including PD-1, TIM-3, CTLA4, and BTLA. Infiltration with FOXP3⁺ T cells has already been shown to support unfavorable prognosis in a variety of malignancies [238]. The majority of FOXP3⁺ TILs in the presented study (chapter 3.4) are not clonally expanded and therefore probably not expanding in response to one or a few specific antigens but rather infiltrate the tumor due to microenvironment-driven cues or are the result of microenvironment-induced differentiation.

Our findings are in accordance with other studies reporting that the majority of rectal cancer patients do not respond to single immune checkpoint inhibition except cases with high mutational load [239, 240]. Whether the mutational load itself, antigen specificity of the T cell clones, or susceptibility to treatment with selected checkpoint inhibitors are responsible for this observation, cannot be concluded from our data. However, we and others have shown that regardless of absolute numbers of mutations, the majority of clonally expanded TILs cannot be assumed to be neo-antigen-specific [5, 142, 235]. The presented data suggest the impressive

effects of immune checkpoint blockade in cancer therapy not to be mediated by direct interference with functional inhibition of tumor-specific T cells but rather result from secondary effects mediated by inflammation, among others. This suggestion is supported by the timeframe of several weeks after beginning of checkpoint blockade until clinical responses become apparent or phenomena like tumor pseudo-progression occur [241]. Independent from target antigens, our data suggest that expanded T cell clones predominantly infiltrating rectal cancer can only in parts be detected in peripheral blood, which should be considered when relying on peripheral blood as a source for T cells potentially targeting rectal cancer. Future studies will help identifying target antigens of clonally expanded TILs and elucidate the mechanisms underlying effective immune checkpoint blockade.

4.6 Implications of the presented findings on multiple myeloma

A memory B cell subset that shares phenotypic characteristics of naïve ($CD24^{\text{lo}}CD38^+$) and memory ($CD27^+$) B cells was expanded in patients with active multiple myeloma [6]. The subset appeared to be phenotypically stable as frequencies did not change after PMA/Ionomycin or TLR stimulation. Furthermore, this subset was not detected expanded in a single patient with regular immunoglobulin light chain ratio or in patients with colorectal cancer. Therefore, the $CD24^{\text{lo}}CD38^+CD27^+$ phenotype can be assumed to result from long-lasting microenvironmental cues such as cytokines predominantly secreted during active disease. With respect to clinical significance, we do not expect B cell immune phenotyping to replace other established measures for multiple myeloma activity such as serum immunoglobulin light chain levels or bone marrow histology. Moreover, additional work is needed to further characterize and possibly detect this B cell population in other malignant or autoimmune diseases.

$CD24^{\text{lo}}CD38^+CD27^+$ memory B cells were not clonally expanded and most likely not related to the corresponding multiple myeloma cells. However, they prompted us to study the

phenotypic range particular multiple myeloma clones can occupy at the single cell level. The methodologies presented in chapter 3.6 identified even normal phenotype B cells to be part of the malignant multiple myeloma clone [4]. The existence of clonotypic B cells in multiple myeloma has been under debate since the early 1990-ies [184-195, 242]. Notably, most of the previous studies have analyzed PBMCs instead of bone marrow B cells, may be subject to PCR and/or sampling bias, and none reach single cell resolution. By specifically sorting single bone marrow B lineage cells, we significantly lowered the detection limit compared to previous studies and exclude bias introduced by phenotype contamination when analyzing bulk populations. Notably, the error rate of index sorting can be expected below 1 % of sorted cells, which is lower than the frequency of clonotypic B cells identified in the presented studies [3, 4]. However, sequencing destroys the cells and aberrant immune phenotypes associated with clonotypic B lineage cells could not be identified, which makes their isolation for downstream analyses impossible. At this point, we cannot draw conclusions regarding the biological significance of these rare clonotypic B cells in our experiments, however, the therapeutic success of CD19-targeting chimeric antigen receptor (CAR) T cells even in presumably CD19⁻ multiple myeloma [185] suggests these cells can be clinically relevant in subsets of patients.

5. Conclusions (Zusammenfassung)

This work is focused on human T and B cell immunology in solid and hematopoietic malignancies. Human immunology has been suffering from insufficiencies to experimentally resolve the virtually unlimited richness of phenotypes and functional states within humans. To at least partially overcome technical limitations, this work presents methodologies for high-dimensional high-throughput determination of lymphocyte differentiation and specificities at the single cell level. The methodologies were applied to rectal cancer and multiple myeloma, which are prime examples for the prognostic impact of tumor-infiltrating T cells or in which the immune system (B cell compartment in multiple myeloma) is part of the malignancy.

The included publications present the identification of characteristic-phenotype rectal cancer-infiltrating T cells and, with help of individually created TCR-recombinant reporter cell lines, define the spatial distribution of T cell target antigens within the colo-rectum. In multiple myeloma, the developed methodologies lead to the identification of a novel-phenotype non-plasma cell B lineage subset that is polyclonally expanded in active disease. Furthermore, the phenotypic range of malignant B lineage clones in multiple myeloma was not restricted to plasma cells but included rare normal-phenotype (memory) B cells.

This work is novel and of high impact for the (human) immunology field as it

- provides the technical basis to study human lymphocyte biology at the single cell level in high-dimensional space, which is relevant beyond the presented studies on regulatory T cells, rectal cancer, and multiple myeloma.
- determines accessibility of rectal cancer-infiltrating T cells in peripheral blood.
- determines the spatial distribution of target antigens of rectal cancer-infiltrating T cells.
- defines the phenotypic range of multiple myeloma at the single cell level providing a potential rationale for the therapeutic effect of CD19-targeting therapies in a presumably CD19⁺ disease.

- determines the default differentiation pathway of the CD45RA⁺ regulatory T cell subset during *in vitro* expansion, which is relevant for the design and adoptive transfer of functionally defined cell products.

6. References (Literaturangaben)

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7. Danksagung

Ich bedanke mich bei meinen wissenschaftlichen Lehrern und Mentoren. Dies sind in chronologischer Reihenfolge Holger Hackstein, Petra Hoffmann und Matthias Edinger, Mark Davis und Thomas Blankenstein, die jeder auf seine Weise zu verschiedenen Zeitpunkten den richtigen Zugang zu mir gefunden und entscheidend zur Entwicklung meiner wissenschaftlichen Persönlichkeit und Selbstständigkeit beigetragen haben.

Ich danke den Mitgliedern meiner Arbeitsgruppe, namentlich Kerstin Dietze, Josefin Garmshausen, Maria Fernanda Lammoglia Cobo und Livius Penter, ohne deren Unterstützung und Glauben in unsere gemeinsame Arbeit viele Projekte nicht möglich gewesen wären.

Des Weiteren möchte ich mich bei meinen wissenschaftlichen und ärztlichen Kollegen für viele Jahre Freude, fröhliche Zusammenarbeit und herausragende Flexibilität bedanken.

Bezüglich klinischer Ausbildung und parallel uneingeschränkter Unterstützung meiner Forschung gilt mein ausdrücklicher Dank Reinhard Andreesen, Joachim Hahn, Daniel Wolff, Bernd Salzberger, Christine Dierkes, Julia Langgartner, Sylvia Pemmerl, Andreas Kahl, Anne Krüger, Ralph Kettritz, Adrian Schreiber, York Kühnle, Bernd Dörken und Lars Bullinger.

Bei meinen Eltern und Geschwistern bedanke ich mich für Ihre prägenden Einflüsse, Weitsicht und bedingungslose Unterstützung.

Abschließend möchte ich mich bei allen Menschen bedanken, mit denen ich auf dem Wege der Musik in Kontakt getreten bin. Das gemeinsame Musizieren hat mich in allen meinen Lebensphasen entscheidend geprägt und unterstützt.

8. Erklärung

§ 4 Abs. 3 (k) der HabOMed der Charité

Hiermit erkläre ich, dass

- weder früher noch gleichzeitig ein Habilitationsverfahren durchgeführt oder angemeldet wurde,
- die vorgelegte Habilitationsschrift ohne fremde Hilfe verfasst, die beschriebenen Ergebnisse selbst gewonnen sowie die verwendeten Hilfsmittel, die Zusammenarbeit mit anderen Wissenschaftlern/Wissenschaftlerinnen und mit technischen Hilfskräften sowie die verwendete Literatur vollständig in der Habilitationsschrift angegeben wurden,
- mir die geltende Habilitationsordnung bekannt ist.

Ich erkläre ferner, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis bekannt ist und ich mich zur Einhaltung dieser Satzung verpflichte.

Datum

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